Changes in Cell Wall Structure During Rhizoid Formation of *Silvetia babingtonii* (Fucales, Phaeophyceae) Zygotes

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CHANGES IN CELL WALL STRUCTURE DURING RHIZOID FORMATION OF
SILVETIA BABINGTONII (FUCALES, PHAEOPHYCEAE) ZYGOTES¹.

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8 Running Title: Cell wall in the rhizoid formation of *Silvetia* zygotes

9

ABSTRACT

We examined the ultrastructure of the cell wall and immunolocalization of alginates using specific antibodies against M-rich alginates and MG blocks during rhizoid formation in fucoid zygotes, *Silvetia babingtonii*. The thallus region on the 24-hour old zygotes was composed of a cell wall made of three layers with different fiber distribution. In the 10-hour old zygotes, three layers in the thallus were observed before rhizoid formation, namely the inner, middle and outer layers. During rhizoid elongation, only the inner layer was apparent close to the rhizoid tip area. Immunoelectron microscopy detected M-rich blocks of alginate on the inner half of the cell wall, irrespective of the layer number in the thallus and rhizoid regions. The MG blocks were seen to cover a slightly wider area than M-rich alginate blocks. This likely indicates an alginate deposition on its mannuronate form close to the plasma membrane before subsequent maturation, and MG-blocks generation, in the wall. Transcriptome analysis was performed using 3 -, 10 -, and 24 h-old zygotes after fertilization to examine gene expression level relating alginate synthesis on this time course. The expression level of two mannuronan C5-epimerase homologs in particular, which have the function of conversion of mannuronic acid into guluronic acid in alginates, was upregulated or downregulated over the course of the examination.

Keywords: cell wall, cryofixation, immunoelectron microscopy, RNA-seq, *Silvetia babingtonii*, transmission electron microscopy

Abbreviations: AF, after fertilization; BAM, brown algae monoclonal antibody; MC5E, mannuronan C5-epimerase; RFCW, Rhizoid-flank cell wall; G, Guluronic acid; M, Mannuronic acid; RTCW, Rhizoid-tip cell wall; TCW, Thallus cell wall

INTRODUCTION

Many seaweeds grow in the intertidal region where environmental factors, including temperature, wave shock, intensity and quality of light, salinity, and drying, are changeable. The cell wall protects the cell structure from physical shocks and unfavorable factors, such as herbivores. Therefore, seaweeds develop characteristic polysaccharides in the cell wall to enhance stress tolerance (Kloareg and Quatrano 1988, Popper et al. 2011). In brown algae, the main components of the cell wall are alginates, sulfated fucans, and cellulose. In the mature sporophyte of laminarialean and fucal species, the weight ratio of alginate is three times and more than half that of cellulose and sulfated fucan (Mabeau and Kloareg 1987, Skriptsova et al. 2012). Alginate consists of 1,4 linked mannuronic acid (M) and guluronic acid (G) residues. M-rich blocks show flexible gels, while G-rich blocks cause rigidity. Accordingly, the M/G ratio relates to the properties of the cell wall, such as gel strength and stretching. Young sporophytes of *Costaria costata* show a high M/G ratio, and the ratio gradually decreases with thallus growth (Wu et al. 2014). The conversion of M residue into G residue is carried out by different mannuronan C5-epimerases (MC5E) expressed in different developmental stages and environmental conditions (Nyvall et al. 2003, Tonon et al. 2008). The alginate biosynthesis pathway in brown algae is not fully resolved, especially the first production of a polymer of M residues (mannuronan) (Michel et al. 2010). It is predicted that, shortly after synthesis, the alginate should consist of M-rich

1 blocks; therefore, the M-rich region might hold the clues to the understanding of
2 alginate synthesis.

3 There are some ultrastructural observations of the cell walls of the brown algae.
4 The results showed that three or more cell wall layers were identified in the apical cell
5 of *Sphacelaria rigidula* (Karyophyllis et al. 2000), the meristematic epidermal cells of
6 *Dictyota dichotoma* (Evans and Horrigan 1972), zygotes of furoid algae (Novotny and
7 Forman 1975, Callow et al. 1978, Bisgrove and Kropf 2001), and the lateral cell wall in
8 gametophytes and sporophytes of *Ectocarpus siliculosus* (Terauchi et al. 2016). The cell
9 wall layers were distinguished by the distribution of the cell wall fibers and the matrix.
10 Terauchi et al. (2016) clarified the quantification of the frequency of the electron-dense
11 fibrils (alginate fibrils) and their junction based on three-dimensional analysis using
12 rapid freezing fixation and electron microscopy and tomography. The results showed
13 that the alginate fibers were distributed more densely and in a more complex
14 arrangement in the inner layer than in the middle layer.

15 Tip-growing apical cells of *Sphacelaria* showed a characteristic cell wall
16 structure. In *Sphacelaria rigidula*, the apical dome of the tip cell had a two-layered cell
17 wall. A very thin amorphous layer (L1) covered the cell, and the fibrous layer (L2)
18 existed just beneath the L1 layer. Other parts of the tip cell and the thallus cells were
19 covered with four layers of cell walls, with L3 and L4 added to L1 and L2
20 (Karyophyllis et al. 2000). In *Sphacelaria furcigera*, bud (branch) formation was
21 observed (Burns et al. 1982a, b). The axial cell wall is composed of four layers (CW1–
22 CW4), but the initial bud region is covered by two newly synthesized layers (CWO and
23 CWI). Burns et al. (1982a) suggested that alginates are abundant in CW2, CW4, and
24 CWI, while sulfated fucans were prominent in CW1, CW3, and CWO. Cell wall

1 structure on the rhizoid tip in fucoid zygotes has been observed (Novotny and Forman
2 1975, Callow et al. 1978, Bisgrove and Kropf 2001). Deposition of the highly sulfated
3 fucan occurs in the tip growth region (Quatrano and Crayton 1973, Quatrano and Steven
4 1976, Callow et al. 1978, Brawley and Quatrano 1979, Torode et al. 2016). However,
5 the changes occurring in the cell wall structure during rhizoid development remain
6 unclear.

7 Different appearances in the multilayered cell wall could reflect differences in
8 cell wall components, which would produce different physicochemical properties. To
9 examine the distribution of polysaccharides on the cell wall, immunoelectron
10 microscopy using anti-alginate antibody (Chi et al. 1999, Nagasato et al. 2010, Terauchi
11 et al. 2012, Terauchi et al. 2016), an anti-fucoidan antibody (Nagasato et al. 2010), and
12 a binding assay using a conjugation of cellulase or alginate lyase-colloidal gold were
13 applied (Nagasato et al. 2010, Terauchi et al. 2016). At the light microscopic level,
14 metachromatic analysis using toluidine blue and periodic acid-Schiff (PAS) was
15 performed to detect alginate and sulfated fucan (Novotny and Forman 1975, Burns et al.
16 1982a). Recently, monoclonal antibodies that recognize the different structures of
17 alginate and sulfated fucan have been produced, and immunofluorescence data on the
18 localization of these epitopes in some brown algal species have been collected (Torode
19 et al. 2016, Linardić et al. 2017, Rabillé et al. 2019, Linardić et al. 2020). Antibodies
20 recognizing different epitopes showed spatial and temporal differences in localization.
21 By analyzing the localization of these antibodies through immunoelectron microscopy,
22 it was expected that we could obtain more useful information relating to the
23 multilayered cell wall of the brown algae.

1 In brown algae, there is little existing information on how the cell wall changes
2 to generate tip-growth from the thallus cell. To address this question, we observed cell
3 wall structure during rhizoid growth in the furoid alga *Silvetia babingtonii* zygotes,
4 using the rapid freezing and freeze substitution technique for transmission electron
5 microscopy (TEM). This method is an effective method for preventing from eliminating
6 the soluble polysaccharides, namely alginate and fucan. In this study, we focused on
7 localization of the alginate by immunoelectron microscopy using antibodies recognizing
8 different epitopes. Moreover, to examine the synthesis regulation of alginate on rhizoid
9 growth, transcriptome analysis using mRNA obtained from zygotes before and after
10 rhizoid germination was conducted.

12 MATERIALS AND METHODS

13 *Materials*

14 Mature *Silvetia babingtonii* (Harvey) E.A. Serrão, T.O. Cho, S.M. Boo &
15 Brawley was collected from September to November 2006-2010 at Charatsunai, and
16 2018 at Botofurina, Muroran, Hokkaido, Japan. The zygote preparation procedure was
17 the same as in the previous paper (Nagasato et al. 2010). The collected mature thalli
18 were brought to the laboratory and wiped with gauze after washing with sterile
19 seawater. They were placed in continuous light ($30\text{-}40\text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 18 °C
20 overnight. Then, the thalli were transferred to a cool chamber at 4 °C for 2 h after
21 darkening with aluminum foil. Adding fresh seawater induced the release of eggs and
22 sperm into the medium. The age of the zygotes was counted from one h after pouring
23 seawater. Zygotes were cultured in PES medium (Provasoli 1968) containing $40\text{ }\mu\text{g}\cdot\text{mL}^{-1}$
24 chloramphenicol at 18 °C in continuous light.

Electron microscopy and Immunoelectron microscopy

Zygotes were cultured on gel support films (ATTO Co., Tokyo, Japan). Before fixation, the films were cut into small triangle shapes less than 1cm. Rapid freezing and freeze substitution procedures were performed according to previous reports (Nagasato and Motomura 2002, Nagasato et al. 2010). Finally, the samples were embedded in a Spurr low-viscosity resin (Polysciences, Warrington, USA). Ultrathin sections were cut using a diamond knife on an ULTRACUT ultramicrotome (Reichert-Jung, Vienna, Austria). Sections were picked up on a formvar-coated one-slot copper grid, stained with EM stainer (Nisshin EM, Tokyo, Japan) and then lead citrate (Reynolds 1963). Observations were performed using a JEM-1011 electron microscope (JEOL, Tokyo, Japan).

Localization of the alginate was examined using antibodies against various alginate blocks (Torode et al. 2016). Nickel grids with sections were incubated with blocking solution (2.5% skimmed milk, 5% normal goat serum, and 0.05% NaN_3 in PBS; 137 mM NaCl, 2.7 mM KCl, 4.9 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) for 30 min at 37 °C and then transferred to anti-alginate antibodies, BAM6 and BAM7 diluted 1:5 with PBS for 1 h at 37 °C. The grids were then washed with PBS three times and incubated for 1 h at 37 °C with a goat anti-rat IgG (whole molecule) conjugated with 10 nm colloidal gold particles (Sigma) diluted 1:20 with blocking solution. After washing with PBS and distilled water, sections on the grids were performed using an EM stainer in this case.

Quantification of colloidal gold particles

To quantify the distribution of alginate in the thallus cell wall (TCW) and rhizoid-tip cell wall (RTCW), the distance between each colloidal gold particle and plasma membrane was measured using ImageJ 64 (NIH, Bethesda, MD, USA). Measurements were conducted for three rectangles with the height of the cell wall from the plasma membrane and 0.75 μm width on the same cell wall region against three zygotes (n=9). The thickness of the cell wall differed in each sample; thus, the length between the plasma membrane and the edge of the cell wall was set as one. The conditions that labeled the density of gold particles within 0.25 μm^2 exceeded 10 (Table 1) were performed in this analysis. The distance of gold particles was assumed to follow a beta distribution because the distance was continuous and ranged from 0 to 1. Because the number of particles differed between the antibodies, the relative density of distance was used to investigate the distributions. The kernel function and bandwidth obtained the function to express the density. Assessment of the function was confirmed by goodness of fit tests (GFT). Beta regression was used to investigate the effects of antibodies (BAM6 and BAM7), and the cell wall regions (TCW and RTCW) used as explanatory variables, on the distance, used as a response variable. Since interactions between these three explanatory variables were unlikely, the analyses were conducted on the respective explanatory variables. All statistical analyses were performed using R software (version 4.0.2) (R Core Team 2020) with the libraries *fitdistrplus* (Delignette-Muller and Dutang 2015) and *betareg* (Cribari-Neto and Zeileis 2010).

RNA-seq and differential expression analysis

Total RNA was extracted from zygotes at 3, 10, and 24 h after fertilization using cetyltrimethylammonium bromide (CTAB) buffer (Pearson et al. 2006). The

1 extract was purified with a chloroform-isoamyl alcohol solution (24:1 v/v). After
2 precipitation with 3 M LiCl, contaminating DNA was removed with an RNase-free
3 DNase Set (Qiagen, Hilden, Germany). Total RNA samples with OD_{260/280} >1.8,
4 OD_{260/230} >2.0, were used for library construction.

5 The cDNA library construction, sequencing with Illumina Novaseq6000 (150
6 bp paired-ends), and generating clean reads by filtering raw reads were performed by
7 Filgen Inc. (Nagoya, Japan). The clean reads were assembled using Trinity software
8 (Haas et al. 2013). The assembled contigs were clustered and filtered out using CD-
9 HIT-EST on CD-HIT suite server (Li and Godzik 2006) with 90% similarity, and the
10 longest open reading frame (ORF) sequences were identified by TransDecoder (v5.5.0)
11 (Haas et al. 2013). Sequences with less than 100 amino acids were discarded. The
12 longest ORF sequences were annotated using the annotation pipeline on Maser
13 (Management and Analysis System for Enormous Reads) platform ([https://cell-](https://cell-innovation.nig.ac.jp)
14 [innovation.nig.ac.jp](https://cell-innovation.nig.ac.jp)) (Kinjo et al. 2018). Clean reads were aligned onto the longest ORF
15 sequences and quantified using Salmon (Patro et al. 2017). Data normalization and
16 identification of differentially expressed genes (DEGs) without replicates were
17 performed using an iterative *deseq* pipeline in the TCC package (Sun et al. 2013). False
18 discovery rates (FDR) ≤ 0.05 were considered as DEGs. The Z-scores based on the
19 transcripts per million (TPM) were calculated using R software (3.6.2; [http://www.R-](http://www.R-project.org/)
20 [project.org/](http://www.R-project.org/)), and the heatmap of genes for the biosynthetic pathway of alginates was
21 drawn using the gplots (3.0.1.2) package. The Bioconductor package topGO version
22 2.38.1 (Alexa et al. 2006) were used for Gene Ontology (GO) enrichment analysis in R
23 software. Over-representative Go terms in up-regulated or down-regulated genes in
24 three comparison conditions (3 h AF vs 10 h AF, 3 h AF vs 24 h AF and 10 h AF vs 24

h AF) were identified by Fisher's exact test in combination with the 'weight' algorithm, using a minimum p-value cut-off of 0.01.

Real-Time qPCR analysis

To analyze the transcriptional expression of DEG, RT-qPCR was performed. Synthesize of cDNA as template was performed by PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan) using total RNA 50 ng in 20 µl reaction mixture according to manufacture protocol. Specific primers for each contig were designed using Primer-BLAST (Table S1). The reaction mixture contained 5 µL SYBR Premix Ex Taq II (TaKaRa), 0.4 µl specific primers (10 µM), and 1 µl cDNA template in a total of 10 µl. qPCR was performed using Eco Real-Time PCR system (Illumina, San Diego, CA, USA). After 95 °C for 30 s, the cycling parameters were 95 °C for 5 s, and 60 °C for 1 min and were performed for 40 cycles. The melting curve analysis was as follows: 95 °C for 15 s, 55 °C for 15 s, and 95 °C for 15 s. Transcript levels corresponding to the comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1 genes (putative mannuronan C5-epimerase) were compared with the transcript levels of the actin gene (actin; comp21090_c0_seq1.p1). The RT-qPCR was performed in triplicate using three RNA samples from each zygote at the ages of 3, 10, and 24 h. The differences in mean relative expression level between the developmental hours (3, 10, and 24 h) were examined by Tukey's multiple comparison test.

Identifying mannuronan C5-epimerase from S. babingtonii assembled contigs and molecular phylogenetic analysis

A reciprocal blast search against 24,486 proteins of the brown algae *Ectocarpus siliculosus* (<http://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2>) was also conducted to identify genes for enzymes in the biosynthetic pathway of alginates using BLAST+ version 2.7.1 (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST>). To construct the MC5E phylogenetic tree, 36 sequences from *E. siliculosus* (<https://bioinformatics.psb.ugent.be/orcae/overview/EctsiV2>), one sequence from *Saccharina japonica* (Inoue et al. 2016), and seven sequences from assembled *S. babingtonii* contigs were gathered. Three bacterial AlgG sequences from *Azotobacter vinelandii* (P70805), *Pseudomonas aeruginosa* (Q51371), and *P. fluorescens* (P59828) were added to the alignment file as an outgroup. Phylogenetic analysis of amino acid sequences of MC5E was carried out using web service NGphylogeny.fr (<https://ngphylogeny.fr/>) (Lemoine et al. 2019). The sequences were aligned using MAFFT with the iterative refinement method and the scoring matrix Blosom62 (Katoh et al. 2002). Ambiguously aligned non-informative regions were removed with BMGE (Criscuolo and Gribaldo 2010). Twenty sequences were removed due to insufficient sequence length or having identical sequences to the others from the phylogenetic analysis. The model test for maximum likelihood (ML) method was carried out by SMS (Lefort et al. 2017), and phylogenetic analysis was performed using PhyML (Guindon et al. 2010). The best-fit model was Blosom62 + G + I + F based on Akaike information criteria (AIC). Bootstrap values (Felsenstein 1985) were obtained from the analyses of 100 pseudoreplicates with Booster (Ehman et al. 2018). The domain searches against the InterPro server (<https://www.ebi.ac.uk/interpro/>) were also carried out.

RESULTS

Immediately after fertilization, the *Silvetia* zygotes showed a spherical shape (Fig. 1a) and rhizoid generation initiated at 12 h after fertilization (AF) (Fig. 1b). The first and second cytokinesis occurred at 20 h and 24 h AF, respectively (Fig. 1c, d). We observed the ultrastructure and the alginate occurrence of the cell walls in the zygotes after 12 h and 24 h AF. Three cell areas were observed in terms of cell wall structures (Fig. 1e): (i) the thallus cell wall (TCW), (ii) the rhizoid-flank cell wall (RFCW), and (iii) the rhizoid-tip cell wall (RTCW).

At 12 h AF, the TCW was filled with an electron-dense structure with parallel-oriented fibrils (Fig. 2a). In the RFCW, three layers with different contrasts, dark-light-dark, were visible (Fig. 2b). The RTCW showed less stainability and irregularly oriented fibrils (Fig. 2c). The cell wall structure in the zygotes at 24 h AF was examined (Fig. 3a). The appearance of the TCW was dependent on the cutting angle of the ultra-thin sections to the cell wall and the plasma membrane. The section by an oblique angle of the cell wall to the plasma membrane revealed three distinct layers with different stainability and fiber-running orientation (Fig. 3b). The cell wall was roughly divided in three equal parts with the inner (IL), middle (ML) and outer (OL) layers representing 31, 35 and 34% of the whole cell wall, respectively. However, it was difficult to define each layer on a perpendicular section of the cell wall to the plasma membrane because of high electron density (Fig. 3c), as in 12 hour-old zygotes (Fig. S1a). In the RFCW, the outer and middle layers were hardly observed (Fig. 3d, e). First, the outer layer is not apparent at the base of the rhizoid tip (the site indicated by a double arrowhead in Fig. 3a), and the middle layer faded away subsequently. The remaining inner layer became thicker closer to the rhizoid tip (Fig. 3f). While well-

1 structured in other regions, the distribution of fibrils was irregular or wavy closer to the
2 tip. The mucilage material covered the outside of the rhizoid. The amorphous
3 mucilaginous layer was not included in the numbering of the cell wall layer in this
4 study.

5 To locate alginate epitopes in the different cell wall regions, immunoelectron
6 microscopy using anti-alginate antibodies was conducted in zygotes at the age of 24 h
7 (Fig. 4). Localization of M-rich blocks detected by BAM6 antibody was found in the
8 inner half of the TCW, RFCW, and RTCW (Fig. 4a, b, c). The labeling density of gold
9 particles using BAM6 on the RFCW within $0.25 \mu\text{m}^2$ was small (Fig. 4b, Table 1),
10 compared with that in the other two regions. MG-alginates, as detected by the BAM7
11 antibody, were more thoroughly distributed along the wall than MM-blocks, and with
12 dense occurrences (Fig. 4d, e, f). The mean value of the density of gold particles using
13 BAM7 was clearly higher in the order of RTCW, RFCW, and TCW (Table 1).

14 The width of cell wall between the plasma membrane and the edge of the cell
15 wall was set as one and divided into ten section. Distribution patterns on the relative
16 distance, ranging from 0 to 1, of gold particles in the cell wall were estimated well by
17 the function fitted at 10 segments (GFT, $P < 0.01$ for all cases) (Fig. S1). The density of
18 particles binding to BAM6 in the control peaked at a distance of approximately 0.3,
19 which was consistent with the boundary between the inner and middle layers. The
20 density distributions of distances were flatter for BAM7, with a range of 0 to 0.8, than
21 for BAM6 in TCW with shifts of peaks from short at 0.3 to long distances at 0.6 (beta
22 regression, $z = -6.193$, $P < 0.001$). These results showed that the gold particles binding
23 to BAM7 were distributed close to plasma membrane compared with BAM6. The
24 relative densities of distance in TCW showed another peak at 0.7 of relative distance.

To understand the genes involved in early embryo development, transcriptomic analysis was performed at three different developmental stages (3 h AF: time of increased wall thickness, 10 h AF: time of initiation of rhizoid germination, and 24 h AF: time that the second cytokinesis finished and the rhizoid elongated) (Fig. S2). The obtained short reads data are summarized in Table S2. *De novo* assembly produced 97,825 contigs. Filtering with CD-HIT-EST reduced the size of the assembly to 78,268 contigs, and the 27,622 longest ORF contigs were detected by TransDecoder. A total of 11,087 contigs (40%) were annotated by BLASTX for UniProt database (Table S3), and 61–63% of reads were mapped to the 25,890 longest ORF contigs. Then, differential expressed genes (DEGs) were detected for 107 contigs in 3 h AF vs. 10 h AF, 47 contigs in 3 h AF vs. 24 h AF, 125 contigs in 10 h AF vs. 24 h AF of the 25,890 contigs (Fig. S3; Tables S4-S6). GO enrichment analysis indicates that sulfotransferase activity (GO:0008146) and extracellular region (GO:0005576) were significant in the down-regulated genes on 24 h AF (Table S7).

Reciprocal best-hit blast against *Ectocarpus siliculosus* reference proteins showed 39.6% (9,699) proteins in *S. babingtonii* longest ORF contigs (Table S7S8). The BLAST search indicated that some alginate synthetic pathway enzymes, two mannose-6-phosphate isomerase (*MPI*), one phosphomannomutase (*PMM*), one GDP-mannose 6-dehydrogenase (*GMD*), and seven *MC5E*, were conserved in *S. babingtonii*. The expression patterns of these enzyme genes are summarized in Fig. S4). The expression levels of *MPI* (comp32248_c1_seq4.p1) and *GMD* (comp32002_c0_seq6.p1) remained stationary throughout the experiment. Five *MC5Es* (comp12459_c0_seq1.p1, comp21318_c0_seq3.p1, comp25876_c0_seq1.p1, comp32408_c2_seq24.p1 and comp32951_c0_seq4.p1) were up-regulated as the

developmental stage proceeded, and one (comp33109_c0_seq1.p1) was slightly suppressed in 24 h AF.

Domain search and phylogenetic analysis of MC5Es to clarify the molecular function were performed. The catalytic MC5E domain. And the signal peptide region was detected in four contigs (Fig. S5). The phylogenetic tree of MC5Es shows five clades supported with high to moderate value (42-100%) and seven MC5Es homologs of *S. babingtonii* were scattered within these five clades (Fig. S5). Two *MC5E* homologs (comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1) were detected as DEGs in different embryo development stages. RT-qPCR was carried out on two contigs (comp32408_c2_seq24.p1 and comp33109_c0_seq1.p1) (Fig. 5). The expression level of comp32408_c2_seq24.p1 stayed lower until 10 h AF and increased eight times at 24 h AF. Comp33109_c0_seq1.p1 expression level declined to one-fifth at 10 h AF from 3 h AF.

DISCUSSION

Ultrastructural comparisons of cell walls on the rhizoid and thallus parts of fucoid zygotes have been reported (Novotny and Forman 1975, Vreugdenhil et al. 1976, Callow et al. 1978, Bisgrove and Kropf 2001). Previous studies have shown that the cell wall of the thallus region consists of two layers, and the rhizoid cell wall structure has a one or two layers (Novotny and Forman 1975, Vreugdenhil et al. 1976, Callow et al. 1978, Bisgrove and Kropf 2001). We observed three layers in TCW in *Silvetia babingtonii* zygotes at 24 h AF. Occasionally, it was difficult to define the number of layers in the TCW, even in the same zygote. The cutting angle of the section affects the appearance of the cell wall structure (Terauchi et al. 2016). To avoid this, we prepared

consecutive serial sections in each zygote and observed the cell wall structure. Bisgrove and Kropf (2001) mentioned a porous area adjacent to the plasma membrane within the thicker inner layer using chemical fixation. Although they did not count it as another layer, we considered that the porous area corresponds to the inner layer observed in the present study using rapid-freezing and freeze substitution (Fig. 3b).

Regarding the rhizoid cell wall, we observed that the two outer and middle layers from the thallus region were not apparent at the RFCW, and only the inner layer surrounded the rhizoid tip. The inner layer became gradually thicker, and the cell wall fibrils showed an irregular orientation close to the rhizoid tip. This observation is the first to explain how cell wall structure changes during rhizoid elongation in fucoid zygotes at the ultrastructural level. We considered that the cleavage of the two outer layers occurred during rhizoid elongation, not during its initiation. In the RTCW of the zygote at 12 h AF, namely, the stage of initiation of rhizoid elongation, the cell wall layer could not be clearly seen (Fig. 2c); however, the three layers were observed in the RFCW (Fig. 2b). At the beginning of tip growth, the three layers extended; however, the outer two layers might break by limitation of extension according to the further outgrowth. On the other hand, the inner layer could be extended by the addition of new cell wall materials and remodeling.

In land plants, an ultrastructure in the tip growth region is observed during root hair and pollen tube growth. In *Arabidopsis thaliana*, the growing root hair cell wall shows a single layer, and the mature one is surrounded by two layers with different textures and orientation of microfilaments (MFs) (Akkerman et al. 2012). A similar pattern has been observed in *Equisetum hyemale* (Emons and Wolters-Arts 1983). Expansin, a protein that promotes cell wall loosening without degradation of cell wall

1 polymers, is involved in root hair initiation and elongation in many land plants (Cho
2 and Cosgrove 2002, Cosgrove 2015). The apical dome of the pollen tube consists of an
3 outer layer with pectin and a translucent inner layer with callose and cellulose in *A.*
4 *thaliana* (Dardelle et al. 2010, Chebli et al. 2012) and *Nicotiana tabacum* (Geitmann et
5 al. 1995, Ferguson et al. 1998). In the apical dome of the outer layer, highly esterified
6 pectin deposits are created by exocytosis, while localization of low-esterified pectin
7 increases from the transition area between the apex and axial region in *A. thaliana*
8 (Chebli et al. 2012). De-esterified pectin is produced by pectin methyl esterase from
9 methyl esterified pectin, and the degree and pattern of methyl-esterification of pectin
10 influence the elasticity of the gel (Willats et al. 2001). In the transition area, remodeling
11 of the cell wall by accumulation of callose and cellulose with de-esterification of pectin
12 occurs, and this affects the aperture of the pollen tube and promotes tip-growth in *A.*
13 *thaliana* (Chebli et al. 2012).

14 It was expected that elongation of the rhizoid cell wall would be caused by
15 supplying the new cell wall material and remodeling of existing components to allow
16 elongation in the zygotes of *S. babingtonii*. We focused on the spatial distribution of
17 alginate because it is the main polysaccharide of the brown algal cell wall and relates to
18 the property of the cell wall. Therefore, immunoelectron microscopy using antibodies
19 against the different epitopes, BAM6 and BAM7, was conducted against the TCW and
20 the RTCW. There was no difference in the distribution of gold particles between the
21 thallus and rhizoid regions. A peak in the gold particle density of BAM6 was observed
22 close to the plasma membrane in the RTCW, which differs from the situation in the
23 TCW (Fig. S1). Alginate synthesis pathways in brown algae are not fully understood.
24 However, it is considered that alginate is initially synthesized as a polymer of

mannuronic acid (mannuronan) by alginate synthase, and a part of the mannuronic acid (M) is converted into guluronate acid (G) residue by mannuronan C5-epimerases (MC5Es) (Michel et al. 2010). In other words, the M residue-rich area would be considered to be the relatively newly synthesized alginate. Quantifying the gold particles using BAM6 showed that the peaks were detected in the inner layer of TCW and RTCW, and this suggests that alginate synthesis takes place at an adjacent site or on the plasma membrane. In this study, the outer layer was labeled as either BAM6 or BAM7. In *Ectocarpus siliculosus*, the alginate lyase conjugated with gold particles binds to the entire cell wall but labeling with an anti-alginate antibody is limited to the inner two layers of the three (Terauchi et al. 2016). Therefore, it was considered that the outer layer of *S. babingtonii* zygotes contained alginate with different types of MG blocks, which are recognized by BAM6 and BAM7. We also performed immunoelectron microscopy using the anti-alginates BAM8 to BAM11 antibodies (Torode et al. 2016), however they were unable to label the outer layer of the cell wall in *S. babingtonii* zygotes.

Transcriptomic analysis was performed in 3-, 10-, and 24-hour-old zygotes of *S. babingtonii* and focused on the gene expression related to alginate modification. The enrichment of term "sulfotransferase activity" (GO:0008146) and "extracellular region" (GO:0005576) indicate some cell wall components including sulfated fucan might be actively modified during rhizoidal growth. By constructing *de novo* transcriptome assembly, we found homologous genes required for alginate synthesis and modification (Michel et al. 2010). Previous genomic or transcriptomic studies revealed that there were 31 MC5E genes in *Ectocarpus* (Michel et al. 2010), 105 genes in *Saccharina japonica* (Ye et al. 2015), and 38 genes in *Sargassum thunbergii* (Liu et al. 2014). In

1 this study, 19 MC5E genes were identified by BLAST for the UniProt database (Table
2 S3), and the number was smaller than that of other brown algae. Because of our RNA-
3 seq data obtained from only the early developmental stage (3-24 h AF), a part of MC5E
4 genes in *S. babingtonii* might not be expressed. Seven *S. babingtonii*'s MC5Es included
5 a common MC5E domain, but each MC5E included a variety of domain structures
6 similar to *Ectocarpus*'s genes (Fischl et al. 2016). This protein domain diversity in the
7 MC5Es family contributes to the regulation of alginate composition and remodeling in
8 the cell wall. Within the data, two *MC5E* homologs (comp32408_c2_seq24.p1 and
9 comp33109_c0_seq1.p1) showed significantly different gene expression in embryo
10 development stages. It is known that MC5E genes show different transcript patterns in
11 different developmental times, regions, and life cycles (Nyvall et al. 2003, Tonon et al.
12 2008, Fischl et al. 2016, Linardić et al. 2020). There have been reports showing that the
13 transcripts required for rhizoid germination were synthesized up to 5 h AF (Quatrano
14 1968, Kropf et al. 1989). From these findings, comp33109_c0_seq1.p1 may work for
15 rhizoid germination, while it is also possible that the gene may be related to the
16 expansion of the cell wall and the formation of the multilayer before rhizoid formation.
17 The expression level of comp32408_c2_seq24.p1 increased in 24 h AF, suggesting that
18 the gene would involve cell wall remodeling for rhizoid elongation.

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Figure Legends

Fig. 1. *Silvetia* zygote development. (a-d) 30 min AF, 12 h AF, 20 h AF, 24 h AF, respectively. Arrows and arrowheads indicate the first and second cytokinetic planes, respectively. (e) Illustration indicating three cell wall regions observed by TEM; the thallus cell wall (diagonal dot line area), the rhizoid-flank cell wall (striped area), and the rhizoid-tip cell wall (plane area). Scale bars=50 μ m (a-d)

Fig. 2. Ultrastructure of (a) TCW, (b) RFCW, and (c) RTCW in zygotes at 12 h AF. Arrows show the outer edge of cell wall. Arrowheads show plasma membrane. IL; the inner layer, ML; the middle layer, OL; the outer layer. Scale bars=500 nm.

Fig. 3. Zygotes at 24 h AF. (a) Fraction of the *Silvetia* rhizoid. A double arrowhead indicates the point from which the two outer layers start to be reduced. (b) Oblique section of TCW. The cell wall consists of three layers. (c) Perpendicular section of TCW. (d) RFCW. The outer layer is disappearing. (e) RFCW. The middle layer fades away. (f) RTCW has only one layer, which is made of rough fibrils. Thick mucilage covers the rhizoid. Arrows indicate the outer edge of cell wall. Arrowheads show plasma membrane. IL; the inner layer, ML; the middle layer, OL; the outer layer. Scale bars=10 μ m (a), 500 nm (b-f)

Fig. 4. Immunogold localization of alginate within cell wall in the zygotes at 24 h AF. (a), (b), (c) shows BAM6 localization. (d), (e), (f) indicates BAM7 localization. (a) (d) TCW, (b) (e) RFCW, (c) (f) RTCW. Arrows show the outer edge of cell wall. Arrowheads show plasma membrane. Scale bars=500 nm (a-f)

Fig. 5. Quantitative analysis of putative *Mannuronan C5-epimerase* genes transcript level in 3, 12, and 24 hour-old zygotes. (a) comp32408_c2_seq24.p1, (b) comp33109_c0_seq1.p1. The relative gene expression was set one at 3 h AF. The error bars indicate SDs of three separate experiments. Tukey's multiple comparison tests were conducted to investigate significant differences between the treatment hours. Asterisks (*) indicate significant differences at $P < 0.01$.

Fig. S1. The distribution of the relative density of relative distance of gold particles binding to BAM6 and BAM7 in TCW and TRCW at 24 h-old zygotes. The relative distance between plasma membrane and the cell-wall edge ranges from 0 to 1. The sum of density is adjusted to 10. Red lines show the function obtained by kernel function and bandwidth.

Fig. S2. Three different embryo developmental stages for transcriptomic analysis. (a) 3 h AF, (b) 10 h AF, (c) 24 h AF.

Fig. S3. MA plots (log expression ratio vs. mean average expression) comparing gene expression during zygote germination. Differential expression genes were shown as magenta points (TCC, FDR < 0.05). (a) 3 h AF vs. 10 h AF, (b) 3 h AF vs. 24 h AF, (c) 10 h AF vs. 24 h AF.

Fig. S4. Schematic representation of the alginic acid biosynthetic pathways with expression levels of putative enzymatic genes during embryo development. The gene

expression level was shown as TPM and Z-score. MPI: mannose-6-phosphate isomerase, PMM: phosphomannomutase, GMD: GDP-mannose 6-dehydrogenase, MC5E: mannuronan C5-epimerase. Asterisks indicate differential expression genes.

Fig. S5. Phylogenetic and putative protein structural analysis of mannuronan C5-epimerases in *S. babingtonii*. Numbers indicate the bootstrap values in the maximum likelihood analysis. Asterisks indicate differential expression genes. Each shape on the schematic protein sequences indicates the catalytic MC5E domain (purple box), a transmembrane domain (cyan oval), a signal peptide region (orange box), and the WSC domains (green box).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. S1

Fig. S2

Fig. S3

Fig. S4

Fig. S5